CORRELATION BETWEEN CYCLIN D1 EXPRESSION AND CLINICAL BEHAVIOUR IN SQUAMOUS CELL CARCINOMA OF THE PENIS

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CERTIFICATE

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DEGREE OF M.Ch. (SURGICAL ONCOLOGY) BRANCH-VII

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DATE

PROF.AND HEAD
DEPT.OF SURGICAL ONCOLOGY

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THIS WORK IS DEDICATED TO CANCER PATIENTS AROUND WHOM

OUR WORK AND LIFE REVOLVES

CORRELATION BETWEEN CYCLIN D1 EXPRESSION AND CLINICAL BEHAVIOUR IN SQUAMOUS CELL CARCINOMA OF THE PENIS

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AIM

To find a molecular marker that would predict the clinical behaviour of the local aggressiveness and their metastatic potential. CYCLIN D1 is a molecular marker that is over expressed in most of the epithelial cancer .So for there are only few studies reporting their significance in penile cancers. From our study we tried to correlate their expression with their clinical behavior.

CHAPTER I

INTRODUCTION AND REVIEW OF LITERATURE

1.1. THE PROBLEM

Penile cancers are uncommon genitourinary malignancies in the western countries. In Europe, the incidence is 0.1-0.9 per 100,000, and in the USA, 0.7-0.9 per 100,000. In some areas of Asia, Africa and South America, the incidence is significantly higher at 19 per 100.000 [1] in these countries, penile carcinoma accounts for as many as 10-20% of male cancers. This high incidence is related to the poor personal hygiene, socioeconomic conditions and the high prevalence of human Papilloma viruses.

In the Madras Metropolitan Tumor registry there were 98 cases reported between 1999 and 2001 for an average total mid year male population of 2,178,842 of which there were 1,643,901 men of 15 – 70 years age group[2]. The crude incidence is 1.5per 100,000 population. The incidence remains stable in most of the western countries, however there is a trend towards a decrease in the incidence in our country due to the improvement in the personal hygiene and socioeconomic status.

Penile cancers are generally slow growing tumors with predictable orderly spread to the superficial and deep inguinal and to the pelvic nodes. Distant metastases at presentation are rare. These cancers have a spectrum of presentation with indolent slow growing lesions to highly aggressive metastatic disease. The survival drops drastically when the patient develops nodal metastases[3]. At present there are few clinical or pathological markers which would predict the aggressiveness of these tumors. No definite molecular marker has been identified to predict the aggressiveness.

1.2. PATHOLOGY AND PROGNOSTIC MARKERS

Penile carcinoma essentially metastasizes via the lymphatic system and develops mainly through the embolization mechanism instead of lymphatic permeation. Distant metastases are very rare and are a result of vascular dissemination [2]. Spreading essentially develops in stepwise fashion; inguinal lymphatic spread occurs first, followed by pelvic metastases, and lastly by distant metastases. As a consequence, it is extremely rare to observe patients with positive pelvic nodes or distant metastasis without inguinal lymph-node involvement. The primary tumor is localized to the glans in 48% of cases, prepuce in 21%, both glans and prepuce in 9%, coronal sulcus in 6%, and less than 2% in the shaft [5]. Palpable inguinal nodes are present at diagnosis in 58% of patients

(range 20-96%) [6]. Of these patients, 17-45% has nodal metastases, while the remaining patients have inflammatory disease secondary to an infection of the primary tumor.

The histological types, grade and growth patterns of penile cancers are

TYPES OF SCC

- Classic
- Basaloid
- Verrucous and its varieties (24):
- Warty (condylomatous) carcinoma
- Verrucous carcinoma
- Papillary carcinoma
- Hybrid verrucous carcinoma
- Mixed carcinomas (warty-basaloid carcinoma, adeno-basaloid carcinoma)
- Sarcomatoid
- Adenosquamous

GROWTH PATTERNS OF SCC

- Superficial spread
- Nodular or vertical-phase growth
- Verrucous

DIFFERENTIATION GRADING SYSTEMS FOR SCC

- Broders system traditionally used as a grading system
- Maiche system score[7] currently seems to be the most suitable grading system

Of all the cancers, squamous cell carcinomas account for more than 95%. The prognostic factors are the T status, the histological grade, and the presence of the nodal metastasis. The histological grade predicts the development of the inguinal metastases to some extent. However substantial number of patients with high grade N_0 disease undergoing nodal dissection will have pathological N_0 disease facing the morbidity of inguinal dissection.

1.3. CLINICAL STAGING

AJCC 2002 TNM TUMOUR STAGING

T - Primary tumour

TX Primary tumour cannot be assessed

T0 No evidence of primary tumour

Tis Carcinoma *in situ*

Ta Non-invasive verrucous carcinoma

T1 Tumour invades subepithelial connective tissue

T2 Tumour invades corpus spongiosum or cavernosum

T3 Tumour invades urethra or prostate

T4 Tumour invades other adjacent structures

N - Regional lymph nodes

NX Regional lymph nodes cannot be assessed

N0 No evidence of lymph node metastasis

N1 Metastasis in a single inguinal lymph node

N2 Metastasis in multiple or bilateral superficial lymph nodes

N3 Metastasis in deep inguinal or pelvic lymph nodes, unilateral or bilateral

M - Distant metastasis

MX Distant metastases cannot be assessed

M0 No evidence of distant metastases

M1 Distant metastases

1.4 SURVIVAL

An overall 5-year survival rate of 52% has been reported. This ranges from 66% in patients with negative lymph nodes to 27% in patients with positive nodes [8, 9] and 0-38.4% in patients with pelvic node involvement. Most patients are elderly and the neoplasm has a slow growth rate. Death from cancer is usually a consequence of local complications, such as infection, haemorrhage of the ulcerated tumour or ulcerated inguinal metastases. The crude 5-year survival rate was 95% for patients with negative nodes, 76% when only inguinal nodes were positive, and 0% when the pelvic nodes were positive. The adverse prognostic factors were (1) involvement of > 3 inguinal nodes, (2) perinodal infiltration in the inguinal area, and (3) pelvic node involvement.[10]

CHAPTER II

MOLECULAR ASPECTS OF CELL CYCLE - THE ROLE OF CYCLIN D1

2.1 CELL CYCLE

Cell cycle is comprised of four phases: g1, s phase, g2 and m phase.

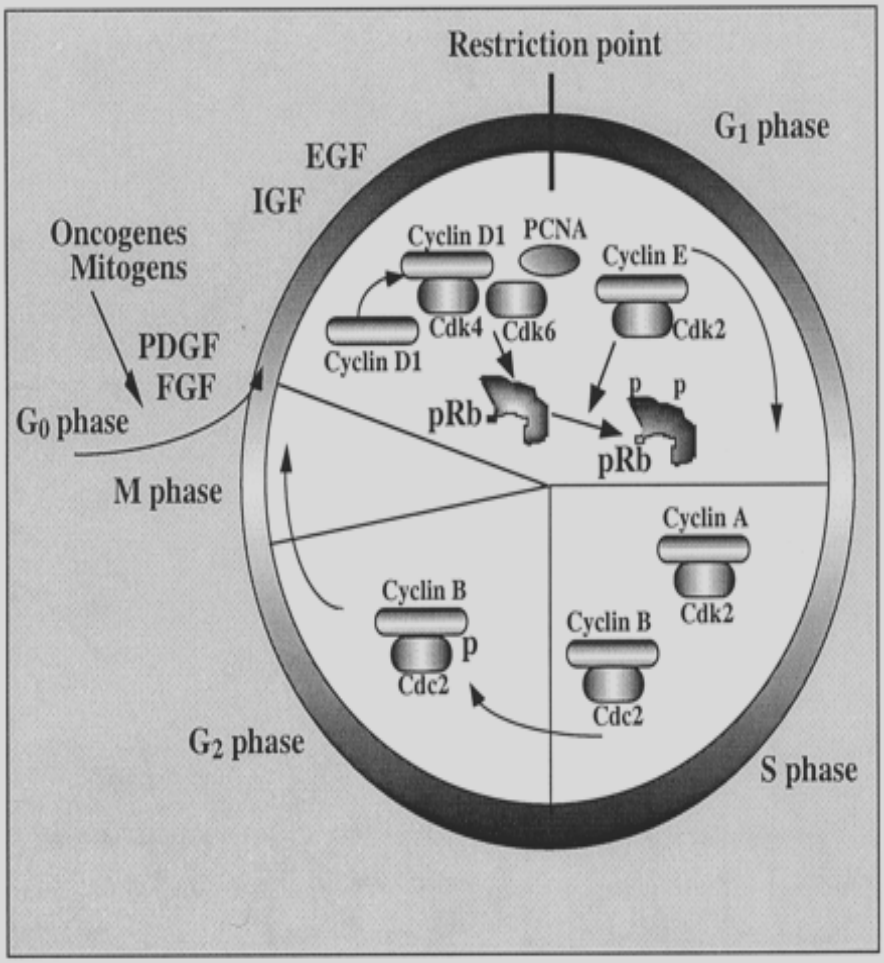
The g1 phase before the mitosis are the times of regulatory decisions of the cycle.(fig 1)

2.2 CYCLIN DEPENDENT KINASES

The major transitions of the eukaryotic cell cycle is triggered by a family of serine threonine kinases called cyclin dependent kinases (cdk) at least nine cdks are known so far [11]. CDK activation requires the regulatory subunits called the cyclins and phosphorylation of a conserved threonine by the cdk activating kinase (CAK) which itself is a complex of a regulatory cyclin H and a catalytic cdk7 subunit , cellular cdk levels tend to remain in constant excess through out the normal cycle and catalytic subunit of cdks are regulated post translationally. Cdk are closely related in size and sequence (> 40 % identity).

In humans, the growing list of cdks include the confounding member, cdc2 and cdk2- cdk7. Our understanding of cdk structure and function (fig-1) is based largely on studies of prototypical cdks of s,pombe(cdc2), s. Cerevisiae (cdc28) and vertebrates (cdc 2 and cdk 2) . The typical cdk catalytic unit

Fig 1



contains 300 aminoacids. The catalytic subunit is completely in active when it is monomeric and unphosphorylated. Cyclins are the primary regulators of the cdks.

p34 CDC 2 (cdk1)

The earliest reported members of the cdk family constitute homologous 34 kda products encoded by the yeast cell division cycle (cdc) genes. p34 cdc2 (cdk1) is a highly conserved cyclin associated 34 kda protein kinase that becomes activated on phosphorylation. Complexed with cyclin b it forms the maturation factor (MPF).Without p34cdc2 the cells are unable to divide[12]. p34 cdc2 distribution is cytoplasmic during the interphase, shifts into the nucleus at the beginning of the prophase and extends through the cell in the mitiotic phase, as the key regulators of the cycle, the cyclin dependent kinases must be tightly regulated by extra and intra cellular signals. The activity of cyclin dependent kinases is controlled by four highly conserved biochemical mechanisms forming a web of regulatory pathways unmatched in this elegance and intricacy.

2.3 CELL CYCLINS

Cyclins are a class of structurally related proteins that bind and activate the catalytic subunit of the CDKs. To data there are eight types of cyclins, (Cyclins A – H) and all of them share an ~ 150 amino acids of the region of homology called the cyclin box which is responsible for the CDK binding and activating[14]. Cyclins can be roughly divided into

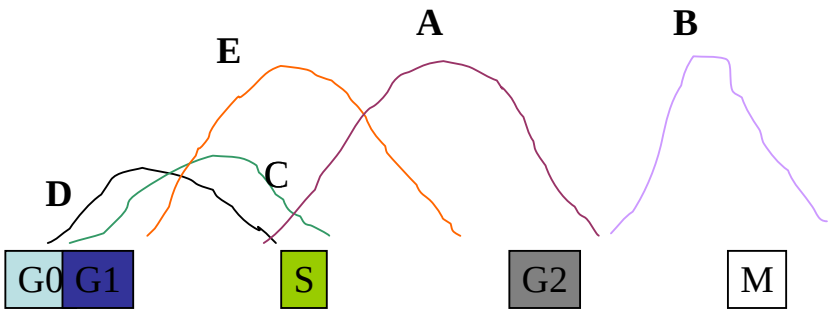
two subfamilies: the G1 cyclins and the mitotic cyclins. The G1 Cyclins (C, D, E) are short lived and have rapid turnover throughout cell cycle(fig 2). Their levels are determined of transcription of their m RNA. The mitotic cyclins (A,B) are very stable throughout the interphase but undergo rapid proteolysis by an ubiquitin dependent pathway during mitosis. Compared to the mitotic cyclins, the G1 cyclins have a longer C terminal sequence after the cyclin box, and it is this part of the protein that seems to confer instability to the G 1 cyclins [14]. The function of cyclins is primarily controlled by changes in the cyclin levels which increase at specific stages and are often categorized by the stage at which they are expressed. The G 1 cyclins differ from the mitotic cyclins in their overall primary structure and this has implications for their stability during the cell cycle. The transcription of the mitotic cyclins, Cyclin A and B are cell cycle dependent and their levels are determined both by transcription and proteolysis[15]. Protein degradation is an effective method for promoting unidirectional cell cycle transitions because of its rapidity and irreversibility. Three major cell cycle transitions, entry into S phase, separation of sister chromatids and exit from mitosis, require the degradation of specific proteins via the ubiquitination by 26S proteosome pathway.

The degradation of mitotic cyclins involves the ubiquitin dependent proteolytic machinery and a small sequence motif called the Destruction box located at the N terminus. This region has a small cluster of conserved residues followed by a lysine rich stretch. The destruction box region differs between Cyclin A and B and probably accounts for the finding that in mitosis Cyclin A is degraded before Cyclin B. G1 cyclins lack the mitotic destruction box but contains the PEST sequences as the peptide motifs that are important for proteolysis. PEST sequences (rich in proline, glutamic acid, serine, and threonine) are frequently present in unstable proteins such as G1 cyclins and contain specific sites of phosphorylation. Phosphorylation of PEST regions facilitates the destructions of PEST sequence proteins. It is not the PEST sequences per se but the specific motifs within them that actually control the PEST sequence recognition by the ubiquitination machinery [16].

Fig 2

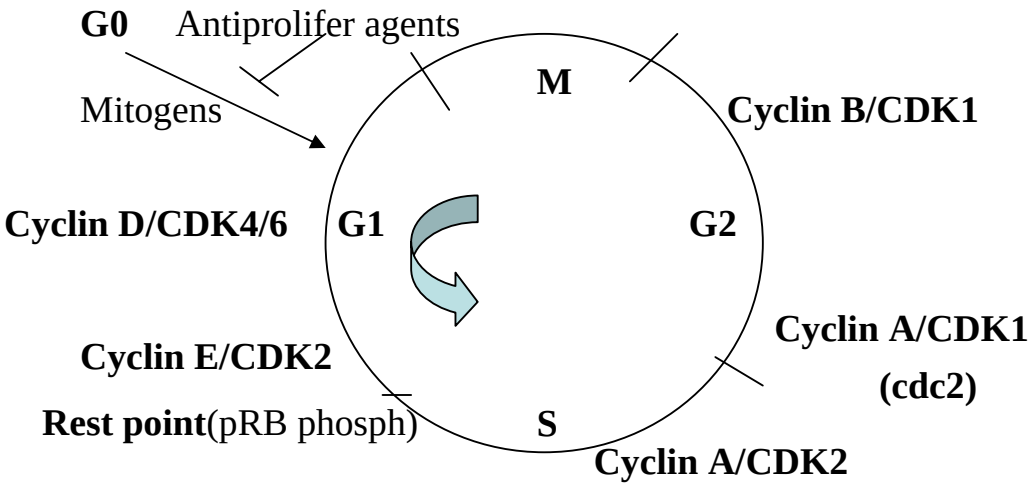
CYCLIN WAVES

Different cyclins/CDK complexes exp at different phases of cell cycle
G1 cyclins---D E A Mitotic cyclin B



Cyclins and cyclin dep Kinases

Regulatory protein subunit-CYCLINS (A-T)
Catalytic subunit(protein kinase)-CDKs (1-9)



Cyclins are thought to target the CDK to specific substrate and subcellular locations. Cyclin expression varies during cell cycle and the periodic expression of different cyclins defines the start of each phase of the cell cycle and also marks the transitions between the various phases. Cyclins and their cognate CDK catalytic subunits non covalently form 1:1 complexes to produce the CDK holoenzyme. Specific CDKs operate in distinct phases of cell cycle. At least two stages within cell cycle are regulated in response to the DNA damage, the G1 – S and the G2 –M transitions. These transitions are called CHECK POINTS at which the cells delay cell cycle progress to allow repair of the DNA damage. Check points are thought to consist of surveillance mechanisms that can detect DNA damage, signal transduction pathways that transmit and amplify the signal to the replication or segregation machinery and possibly repair activities [17].The timing of all the events that transpire during the cell cycle are controlled , through protein phosphorylation.

Table 1 :- Cyclins and cell cycle phases.

S. No	Cyclin	Cell Cycle Phase
1	CYCLIN D1	EARLY G1 phase
2	CYCLIN E	G1 / S PHASE TRANSITION
3	CYCLIN A	S PHASE
4	CYCLIN B	G2 / M PHASE

2.4 CYCLIN D

The first groups of cyclins that are expressed after the cells are stimulated to enter the cell cycle are the D cyclins. D type cyclins act as growth factor sensors. Cyclin D has a relatively short half life of 20 min. approximately and rapidly disappears with the removal of mitogenic stimuli or the addition of antiproliferative agents.[18] Cyclin D complexed with respective partners CDK4 and CDK6, participates in the transduction of external signals (mitogenic or proliferative) to other components of G1 / S transition cell machinery. Cyclin D helps in moving G0 cells into G1 And early G1 Cells into the G1 /S transition in response to the extra cellular stimuli. The D type cyclin belongs to a distinct subset within cyclin family based on structural and functional

criteria. There are three genes identified under this family, Cyclin D1, D2 and D3 [19].

Table 2 Gene Locations Of Cyclins

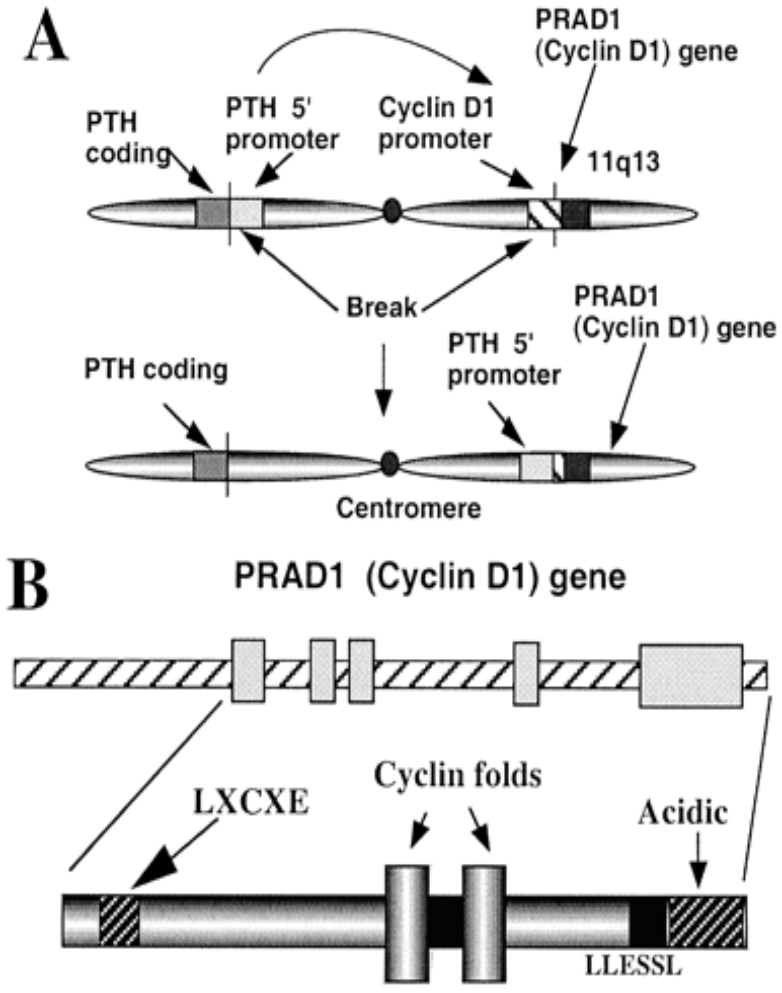
GENE	CHROMOSOME LOCATION	RNA	PROTEIN
CCD1 (CYCLIND1)	11q13	4.5 KB	295AA
CCD2 (CYCLIN D2)	12p13	7KB	289AA
CCD3 (CYCLIN D3)	6p21	2.2KB	292AA

The chromosome 11q13 has been identified as a site of tumor specific translocations .In the parathyroid adenomas, due to clonal inversion, the cyclin D1 gene on chromosome 11q13 is juxta posed on chromosome 11q15. This results in an increase in Cyclin D1 expression. [20,21] Frequent rearrangements of 11q13 has been noted in B cell lymphomas and multiple myelomas. The same locus is subjected to translocation[22], retro viral insertion, gene amplification by yet poorly defined mechanisms in lymphomas, squamous cell carcinomas and breast carcinomas implying that Cyclin D1 acts as a Oncogene –positive growth regulator.

2.5 CYCLIN D1 GENE

The cyclin D1 gene (CCND1), linked closely to the bcl-1 gene on chromosome 11q13, is sometimes referred to as the PRAD1 gene because of an initial finding of frequent rearrangement of this gene in benign parathyroid adenomas.[49] The important role of cyclin D1 in parathyroid neoplasia has subsequently been confirmed[50]. The bcl-1 locus, so called because of the involvement of this chromosomal region in translocations {(t11; 14) (q13, q32)} characteristic of certain B cell lymphomas (now called mantle cell lymphomas), was originally thought to be identical to the cyclinD1/PRAD1 gene, but it has been shown to reside 110–130 kb upstream or centromeric to the PRAD1 gene[51]. However, no transcriptional units have been identified in the immediate vicinity of the major translocation cluster (MTC) [52] of the bcl-1 breakpoint area or any of the breakpoints that have been found up to 63 kb telomeric to the MTC region[53]. The absence of CpG islands between the original bcl-1 locus and PRAD1's CpG Island [51 52 54] lends further support to the notion that no other gene lies within this interval. Thus, the probability remains that the cyclin D1 / PRAD1 gene is the bcl-1 Oncogene. Although bcl-1 often co-amplifies with other genes on 11q13 (such as EMS-1, FGF3, FGF4, int-2, and hst-1), cyclin D1 and EMS-1 are the only proteins, so far, over expressed as a result. [52 55 56]

Fig 3


A
PRAD1
(Cyclin D1) gene
PTH
coding
PTH 5'
promoter
Cyclin D1
promoter
11q13
Break
PRAD1
(Cyclin D1) gene
PTH coding
PTH 5'
promoter
Centromere
B
PRAD1 (Cyclin D1) gene
LXCXE
Cyclin folds
Acidic
LLESSL

The D type cyclins contains the sequence LEU-X- Cys - Glu near their aminoterminus (fig3). This sequence is common to DNA vital oncoproteins SV40 T antigen, adenovirus E1A and human Papilloma virus E7 that bind and activate pRB and pRB related proteins. The D type Cyclins bind directly to pRB and p107 invitro and the interactions are disrupted by point mutations of the LEU-X- Cys - Glu motif. The Oncogene derived peptides that contain this motif compete with Cyclin d to bind to pRB[23]. Cyclin D2 and D3 form more stable complexes than cyclin d1 suggestive of differences in functional interactions not being equivalent[24]. The functional interaction of Cyclin D with pRB underscores its role as a positive growth regulator with Oncogenic potential.

2.6 CELL CYCLE IN NEOPLASIA

Predictably, proteins involved in driving the cell cycle, such as cyclins, are frequently over expressed in primary tumors, whereas proteins that slow cell division, such as the CKIs, are often inactivated. Of the many cell cycle regulators implicated in the development of cancers, cyclin D1 is among the most prevalent. Over expression of D-type cyclins has been shown to contract the G1 phase, decrease cell size, and reduce the dependency of the cell on mitogens [25-27] in animal models and cell lines.

A link between D-type cyclins and the retinoblastoma protein

The connection between D-type cyclins and tumorigenisis is bolstered further by compelling evidence that D-type cyclins are important in cell cycle regulation of the retinoblastoma tumor suppressor protein (pRB), an approximately 105 kDa nuclear phosphoprotein.[28-30].The amount of pRB is not altered with progression of the cell cycle, however, the phosphorylation state of pRB is cell cycle dependent[31-33] . pRB is hypophosphorylated throughout G1 phase, phosphorylated just before S phase, and remains phosphorylated until late mitosis. Hypophosphorylated pRB arrests cells in G1,[34]an effect most likely mediated through complex formation with DNA binding proteins (including members of theE2F family)[35-37] required for transcriptional activation of cellular genes. Phosphorylation of pRB during late G1 phase reverses the growth suppressive effects of pRB, by untethering E2F from its inhibitory constraint and thereby allowing the activation of genes required for DNA replication.[38]Because D-type cyclins are able to bind to pRB through an N-terminal LXCXE motif, [23,39,40]they are excellent candidates for G1 phase pRB protein kinases as part of a complex with their specific Cdk partners. Interestingly, this LXCXE motif is common to the SV40 T antigen, adenovirus E1A, and human Papilloma virus E7 proteins,[38]whichmay also bind to pRB and release E2F; a fact that in part

explains the oncogenic potential of these viruses. Support for the idea that D-type cyclins can inactivate pRB comes from reports that increased amounts of D-type cyclins can reverse the pRB induced cell cycle arrest and accelerate progression through G1[27 39 41 42].

Cells that lack functional pRB have significantly lower levels of Cyclin D1 and Cyclin D1 – CDK [43-46] a result which has been interpreted to mean that hypophosphorylated pRB is involved in the stimulation of Cyclin D1 transcription. The reported ability of exogenously expressed pRB to induce cyclin D1 is in concordance with this hypothesis .[47] Thus a negative feed back loop seems to exist in which Cyclin D1 synthesis and activation lead to pRB phosphorylation , which in turn causes decreased Cyclin D1 expression. [48]

2.7 CYCLIN D1 EXPRESSION IN HUMAN CANCERS

In addition to parathyroid adenomas, increased cyclin D1 expression has been shown in a number of primary human tumors and cell lines. In general, primary tumors provide more reliable information as it is often difficult to determine when the amplification occurred during the development of the cell line. This is because other influences, which cause the cells to proliferate at faster rates, may up regulate cyclin D1 expression. Although increased cyclin D1 protein expression

correlates in most instances with amplification of the CCND1 gene, this is not always the case. In some tumors there is a increased cyclin D1 RNA and/or protein without apparent gene amplification, suggesting that other cellular genes (such as the retinoblastoma gene) may impact on the protein expression of cyclin D1,[57] although all the mechanisms have not yet been satisfactorily elucidated. DNA amplification is the most frequent abnormality affecting the CCND1 gene. Furthermore, no major abnormality in the coding region of the cyclin D1 gene has been detected [58,59] suggesting that it is the normal gene product that contributes to tumorigenesis.

BREAST CANCERS

About half of all invasive breast cancers [76-79] have raised expression of cyclin D1 compared with normal epithelium, although the figure for gene amplification averages around 13%. [57] Some earlier studies[80-83] failed to find any significant association between 11q13 amplification and oestrogen receptor positive cancers, but a number of materials [84–93] has now accumulated supporting a correlation between cyclin D1 gene amplification and protein over expression with oestrogen receptor positive tumors. Studies in mice [94 95] and man [96] link cyclin D1 to steroid induced proliferation of mammary epithelial cells. In fact, cyclin

D1 appears to be an independent activator of the oestrogen receptor. [97] Despite the correlation with oestrogen receptor status, there is lack of agreement as to the prognostic significance of cyclin D1 in breast cancers in general. More work need to be done to identify the subsets of patients in whom cyclin D1 may play a more prominent role. It seems that cyclin D1 is more important in node positive,[74] well differentiated, and particularly lobular, varieties than other types of invasive breast cancer.[91-93]The role of cyclin D1 in ductal carcinoma in situ (DCIS) [98], high grade lesions [99] were more likely to show gene amplification but demonstrated lower percentages of nuclei expressing cyclin D1 protein than low grade lesions, which suggests that mechanisms other than gene amplification may be responsible for increased cyclin D1 protein. In this situation, assessment of cyclin D1 protein in combination with pRB may provide more useful information.[100-102] The over expression of cyclin D mRNA, determined by in situ hybridization, was able to distinguish DCIS from atypical ductal hyperplasia and other lesions associated with a low risk of progression to invasive carcinoma[103].

In patients with ER-positive tumors, high levels of cyclin D1 mRNA were associated with increased risk of relapse, local recurrence, metastasis, and death. There were no clinical correlations with cyclin D1 expression in ER-negative disease. In patients who received endocrine

therapy for their primary or recurrent breast cancers, there was an apparent association between a high cyclin D1 mRNA level and shorter response duration within the ER-positive subgroup. These findings indicate that over expression of cyclin D1 mRNA correlates with a worse prognosis within the ER-positive breast cancer phenotype and may be a contributing factor to the development of endocrine resistance in ER-positive disease[104].

CYCLIN D1 IN PANCREATIC TUMORS

Overexpression of cyclin D1 was identified in 43% of cases, and no correlation was observed with clinical phenotype. The novel observation of frequent over expression of cyclin D1 suggests that this established Oncogene might be implicated in the pathogenesis of human Pancreatic Endocrine Tumors. The absence of detectable alterations in cyclin D1 genomic structure suggests that the mechanism for its Oncogenic activation in PETs may be transcriptional or posttranscriptional[105].

HEAD AND NECK SQUAMOUS CELL CARCINOMAS

A range of 35% to 64% of head and neck squamous carcinomas [106-111] (squamous carcinomas in the oral cavity, nasopharynx, pharynx, hypopharynx, and larynx) show over expression of cyclin D1 and/or CCND1 amplification. Over expression of cyclin D1 in the initial surgical

specimens corresponds not only with more frequent recurrence [109] but also with more advanced disease, lymph node involvement, and reduced overall survival. [107] [110] In one study of squamous carcinomas of the larynx, a correlation among cyclin D1 gene amplification, mRNA over expression, and tumor progression, was shown in a cohort of 46 patients. [107] The above and another study [110]demonstrated a significant association between molecular abnormalities of the cyclin D1 gene and pathological measures of poor prognosis. Recently, over expression of cyclin D1 protein in resected material from head and neck squamous carcinomas was found to be an independent prognostic factor [111] a finding that has been confirmed. [112] In Japanese hypopharyngeal squamous cell carcinomas, cyclin D1 gene amplification and protein over expression correlated not only with prognosis but were also useful in identifying optimum treatment regimens. [113] Cyclin D1 negative tumors responded particularly well to multimodality treatment in these tumors.

ESOPHAGEAL CANCERS

In approximately 30% of esophageal cancers, amplification [113] and over expression [114–116] of cyclin D1 have been demonstrated, with several studies showing an association with increased mortality. [116–118] Also, the ability of antisense to cyclin D1 to reverse the transformed phenotype of

esophageal cancer cells [119] provides strong supporting evidence for the molecule's role in cancers at this site.

Cyclin D1 mRNA was overexpressed in the cytoplasm of cancer cells that showed cyclin D1 gene amplification by Southern blot hybridization**.** Cyclin D1 antigen was overexpressed in the nucleus of these cancer cells. The distribution of cyclin D1 mRNA-positive cells was similar to that of cyclin D1 antigen-positive cells in the cancer tissues. The overall 5-year survival of patients with strongly staining tumors was significantly lower than that of patients with weakly or non staining tumors (7 versus 59%; $P < 0.01$). There was no significant correlation between cyclin D1 expression and other clinic pathological factors. These results suggest that cyclin D1 may play an important role in carcinogenesis and that cyclin D1 over expression may be a useful prognostic factor in esophageal cancer[120].

HEPATOCELLULAR CARCINOMAS

Amplification and raised protein concentrations have been observed in 10% of hepatocellular carcinomas. [121, 122] Among other hypothesis, it has been suggested that hepatitis B or C viral integration within the cyclin D1 gene or one of its adjacent regulator sequences may be a mechanism in malignant transformation. [123] In some reports [124 125] the

hepatitis B viral genome was detected on chromosome 13 at an upstream site close to the CCND1 gene. Another study[122] showed that cyclin D1 amplification was restricted to hepatocellular carcinomas that contained the hepatitis B or C virus. Although the evidence is scant, the possibility of an interaction between these viruses and cyclin D1 is an intriguing one.

COLORECTAL CANCERS

Initial reports [126] using cell lines suggested that cyclin D1 was not an important factor in colorectal adenocarcinomas, recent work has shown not only does cyclin D1 over expression occur as an early event in tumor progression, it may also be an independent prognostic factor.[127] There is increased nuclear immunostaining in adenomatous polyps and adenocarcinomas, but not in adjacent normal, transitional or hyperplastic mucosa. These findings apply to both sporadic [128] and familial forms [129] of colon cancer. Furthermore, as has been demonstrated in the oesophagus, antisense to cyclin D1 inhibits the growth and tumorigenicity of colon cancer cells.

GENITOURINARY CANCERS

Amplification of 11q13 has been demonstrated in between 6% and 21% [130–132] of transitional cell cancers of the urinary bladder, although nuclear accumulation of the protein appears in a much greater percentage of

cases. Alterations in cyclin D1 appear to be an early event in tumorigenesis of the urinary bladder, but the prognostic significance of amplification and over expression remain to be determined. In one study a significant relation between cyclin D1 over expression and low tumor grade as well as T classification were observed, [133] these findings were not found in another study. [134] Abnormalities of cyclin D1 are also common in both vulval and cervical squamous cell carcinomas. [135] At these sites, cyclin D1 appears to inactivate pRB in a similar manner to oncogenic human Papilloma virus genotypes. Thus, it seems that in vulval and cervical squamous carcinomas, human papilloma virus proteins can circumvent cellular requirements for cyclin D1 [136] or vice versa. In endometrial carcinomas, 11q13 amplification is exceedingly rare, but about 40% of cases show aberrant accumulation of cyclin D1. [137] Again, the effect of other genes is the likely explanation for this phenomenon, al-though in this case the interaction of cyclin D1 with p53 [138] appears to be more important than with pRB. Because cyclin D1 can activate the oestrogen receptor independently, [98] if this molecule were also over expressed in endometrial hyperplasia it would provide a useful link with known pathogenetic mechanisms. In epithelial ovarian cancers, abnormalities of cyclin D1 are early events in the progression to malignancy, and they may be associated with the degree of

transformation.[139]There is a strong positive correlation between cyclin D1 and c-K ras immunoexpression, [140] but no correlation with the c-erb-B2 oncogene. [141] The relation with the Ras proto-oncogene is important because it has been shown that inactivation of Ras in cycling cells causes a decline in cyclin D1 protein, accumulation of the hypophosphorylated, growth suppressive form of pRB, and G1 arrest. [142] [143] Strangely, the relation between cyclin D1 amplification and oestrogen is not as clear cut as has been demonstrated in the breast. [141]

LUNG CANCERS

Studies have shown a higher frequency of bcl-1 gene amplification in squamous cell carcinomas of the lung than in other types of non-small cell lung cancer, [145] and an association with poor grade and high Ki-67 labelling index. [146]However, current research is contradictory as to the prognostic usefulness of detecting cyclin D1 protein over expression. [147–148] From studies of the resection margin epithelia of lung cancer patients, com-pared with non-smoking controls, it seems that genetic alteration of cyclin D1 is an early event in non-small cell lung cancer, [148] and therefore an attractive therapeutic target.

SKIN CANCERS

Compared to normal skin and benign lesions, cyclin D1 protein expression is significantly greater in various malignant skin tumors, including squamous cell carcinomas, melanomas, and malignant fibrous histiocytomas. [149] Studies of chemically induced squamous cell carcinomas in mice also implicate cyclin D1 (and other G1 cyclins) in the process of carcinogenesis. [150]

SARCOMAS

Amplification of the cyclin D1 gene has been detected in a small percentage of a variety of sarcomas, [151] but the increased cyclin D1 protein expression in at least some cases may due to a mutant protein with greater stability. [152]

MANTLE CELL LYMPHOMAS

All or almost all mantle cell (centrocytic) lymphomas in several studies[51 60–67] have raised activity of cyclin D1, even in cases in which no rearrangement at 11q13 was found. [68] Generally, however, positive nuclear staining with monoclonal antibody to the cyclin D1 protein correlates with amplification of the CCND1 gene as well as mRNA. [69] It has been suggested that expression of cyclin D1 by

lymphocytes in the mantle zone impairs the capacity of these cells to exit the cell cycle and to differentiate into mature plasma cells. [70] This pathogenetic theory is contradicted by a recent finding of cyclin D1 protein expression in 26% of plasma cell neoplasms, however, the same study [71] supports a relation between mantle cells, plasma cells, and their corresponding neoplasm. An international lymphoma consensus [72]acknowledged the importance of chromosome 11q13 translocation and increased cyclin D1 expression in mantle cell lymphomas; in the future it may be elevated to a defining characteristic, given the high sensitivity and relative specificity of this molecule in mantle cell lymphoma compared with other B cell neoplasms. [61] [73] Cyclin D1 protein expression and bcl-1 gene rearrangement has been identified as a key component in the diagnosis of the blastoid variant [74] of mantle cell lymphoma as well as in an entity closely related to mantle cell lymphoma multiple lymphomatous polyposis.[75]

OTHER SITES AND MALIGNANCIES

Central nervous system malignancies such as astrocytomas [153] and glioblastomas [154] are not exempt from cyclin D1 amplification or protein over expression, nor are gastric adeno-carcinomas,[155] [156]

pancreatic adenocarcinomas (may be associated with a poor prognosis), [157]or squamous carcinomas of the gall bladder. [158]

Only a few human tumors are still holding out against the storm of cyclin D1 research. These include pituitary tumours,[159] renal cell tumors, prostate carcinoma, [160] and many haemopoietic malignancies. However, with some of these tumors there are possible links with cyclin D1. For example, the 11q13 region (although apparently not the CCND1 gene) is important in pituitary neoplasms of MEN-1, and cyclins including cyclin D1 are important in renal development.

In addition to the *MEN1* tumor suppressor gene, the cyclin D1 oncogene has demonstrated a role in the pathogenesis of parathyroid and gastroenteropancreatic neuroendocrine tumors. Upregulation of cyclin D1 is observed early in tumor formation, implying a possible role in tumor initiation. Overexpression of cyclin D1 in the parathyroid glands of mice resulted in the tandem regulation of cellular proliferation and hormonal secretion, a feature intrinsic to neuroendocrine tumors.[161]

Cyclin D1 (CCND1) is a key cell cycle regulatory protein, the over expression of which is often found in human tumors and is associated with cell proliferation and poor prognosis. A common adenine-to-guanine substitution polymorphism (A870G) in the *CCND1* gene

results in an altered messenger RNA transcript and a longer-life protein, which are preferentially encoded by the A allele. The *CCND1* 870A allele may be associated with colorectal cancer, and particularly with forms of the disease that result in severe morbidity and mortality[162].

2.8 CYCLIND1 AND MOLECULAR ALTERATIONS IN PENILE CANCER

Alterations pointing to a disturbed p16 INK4A /cyclin D1/Rb pathway are commonly present in penile carcinomas[144]. Activity of high-risk HPV and the resulting increase in p16 INK4A expression was the most frequently detected mechanism amongst the mechanisms that were analyzed, followed by p16 INK4A hypermethylation and BMI-1 over expression. In addition, there is evidence that penile carcinomas being etiologically heterogeneous, with only a proportion of cases attributable to HPV infection. These results are important when HPV-based immunotherapeutic interventions are envisaged, because in contrast to patients with cervical carcinoma, only about 26% of patients with penile carcinoma are likely to benefit from these vaccines.

CHAPTER III

MATERIALS AND METHODS

Penile cancer patients reported to our institute between the year 1998 and 1999 who were accepted for treatment were chosen for analysis. The data were collected from the case records of the Tumor registry .There were 104 cases of carcinoma penis that were accepted for treatment in our institute between the year1998 and 1999. These records were analyzed.

3.1 PATIENT SELECTION

Of the 104 cases who were accepted for treatment at our institute, those patients who had not completed the treatment were excluded from the study. Those who lost to follow up and histology other than squamous were also excluded from the study.Of those records, 37squamous cell carcinoma patientsof different grades were chosen .The inclusion criteria are – completed treatment , regular follow up , squamous cell carcinoma any grade , complete pathology is available with the paraffin blocks .

3.2 DATA COLLECTION

Case records of those patients who fulfilled the above criteria were taken for analysis. The relevant data were taken from the case records and entered into the database for analysis.

3.3 SAMPLE COLLECTION

The pathology reports of these patients included for analysis were taken and their corresponding paraffin block numbers were taken. These blocks are then retrieved from the pathology department paraffin block bank. Five micron sections are taken from the paraffin blocks and incubated overnight. Suitable positive and negative controls are also taken simultaneously from the previously Cyclin D1 positive sections from the laryngeal cancer .After overnight Incubation the slides were submitted foe immunocytochemistry with Cyclin D1.

3.4 IMMUNOCYTOCHEMISTRY

The paraffin embedded sections of 5 μ were placed on the APES coated slides.The slides were treated in Xylene consecutively twice for 8 minutes each for dewaxing the sections. This was followed by treatment in 100% alcohol consecutively twice for dehydration. The slides were then washed in tap water for 8- 10 minutes, taking care not to disturb the tissue sections . Followed by tap water wash , the slides were then place in 0.3% hydrogen peroxide for 30 minutes to quench the endogenous peroxidase activity in the tissues .

3.5 ANTIGEN RETRIEVAL

Antigen retrieval is needed in the study in order to expose the epitopes for immune reaction .Antigen retrieval was done using10 mM Citrate buffer using , with wet autoclaving at 121° C in 10mM Citrate buffer pH6. After antigen retrieval the slides were cooled and washed with phosphate buffered saline for 5 min.

3.6 BLOCKING AND ANTIBODY REACTIONS

The slides were arranged on a moist chamber, to prevent drying of the sections and 100 μ of 2% Bovine Serum Albumin (BSA) was added to each section, taking care to cover the whole section and left undisturbed for 30 min. Following gentle tipping of BSA off the slides , the primary antibody (75 μl) in 1: 100 dilutions was added to each sections .Care was taken to omit the negative control. The slides were left overnight undisturbed to incubate the primary antibody.

The slides were washed with PBS thrice for 5 minutes each to wash off the excess of primary antibody and secondary antibody (75 μl) was added to each slide and incubated for 35 minutes. The slides were washed in PBS thrice for 5 minutes each followed by addition of ABC (Avidin – Biotin Complex -75μl) onto each slide and incubated for 30 minutes.

3.7 TREATMENT WITH CHROMOGEN DI-AMINOBENZEDENE

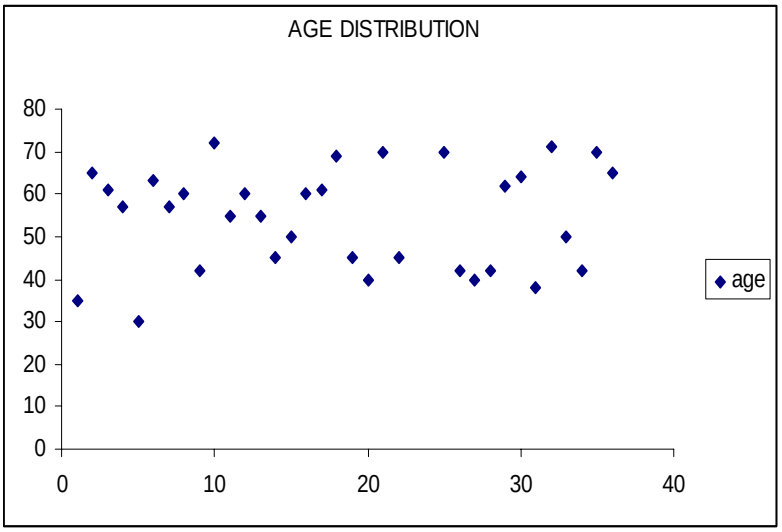
The slides were washed in PBS thrice for 5 minutes each to wash excess of ABC and the slides were treated in a solution of DAB (150 ml of water + 150 ml PBS+100 μl hydrogen peroxide + 150 mg of DAB) for 5 minutes . The excess DAB was washed off the slides in tap water for 5-10 minutes and counterstained in haematoxylin for 2 minutes.

CHAPTER IV

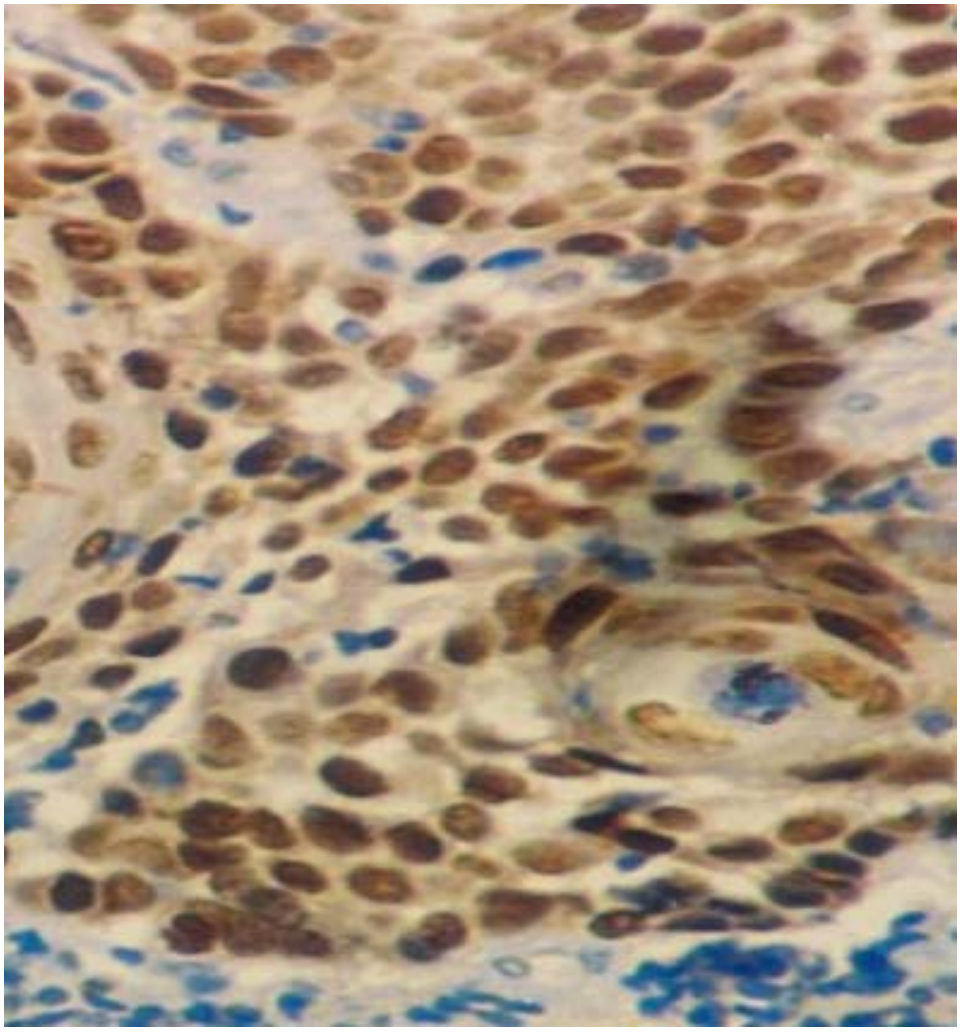
ANALYSIS

The stained slides were the scored by the pathologist. Only the nuclear staining was considered as positive. All cytoplasmic and other nonspecific staining were not considered as positive. Since the adjoining normal squamous epithelium did not over express Cyclin D1 and the corresponding squamous epithelium of the cervix , were > 5 % of expression is considered positive , the same principle was applied here . Depending on the staining intensity, the same is scored as +, ++, +++.

The age distribution were

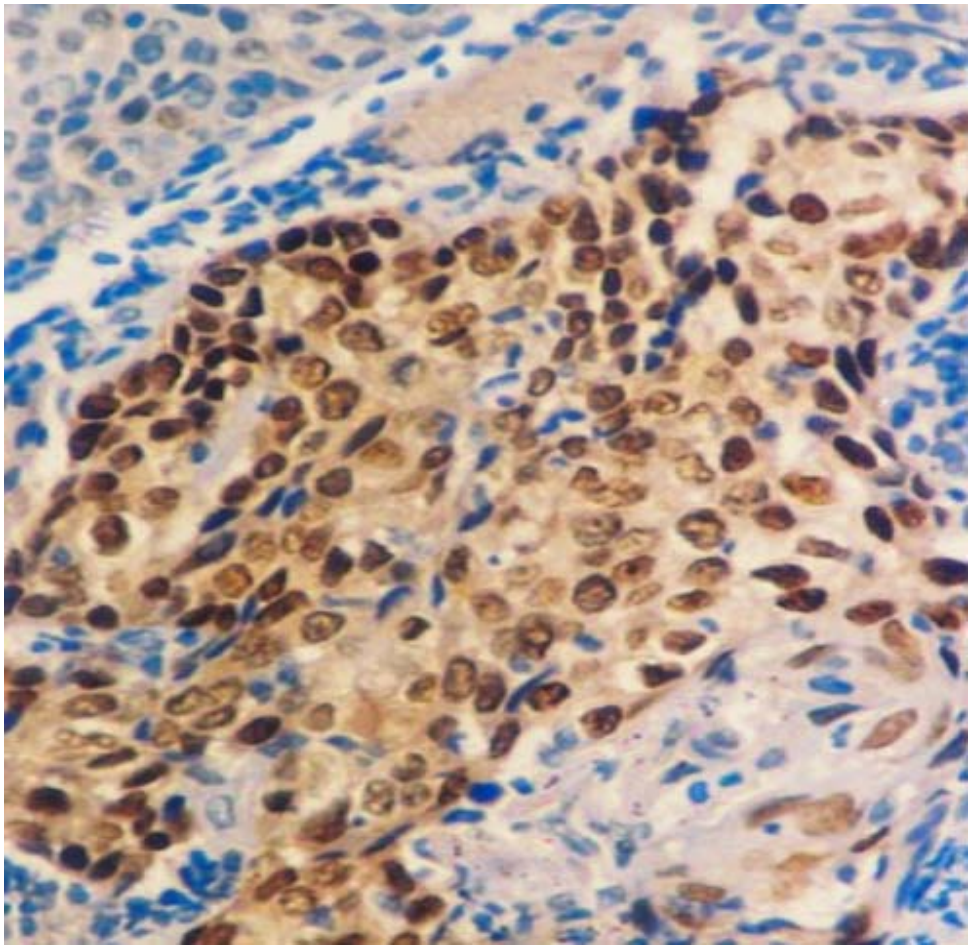


SLIDE SHOWING POSITIVE CONTROL

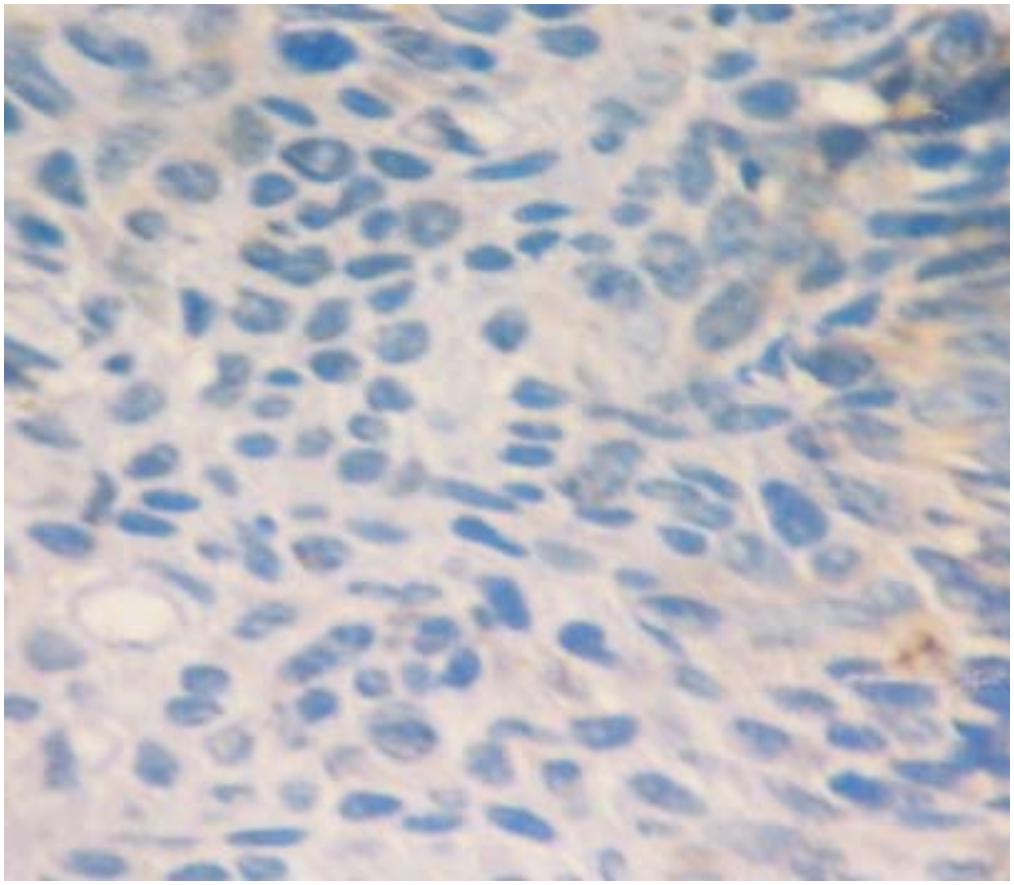


SLIDE SHOWING PENILE CANCER WITH PREDOMINANT

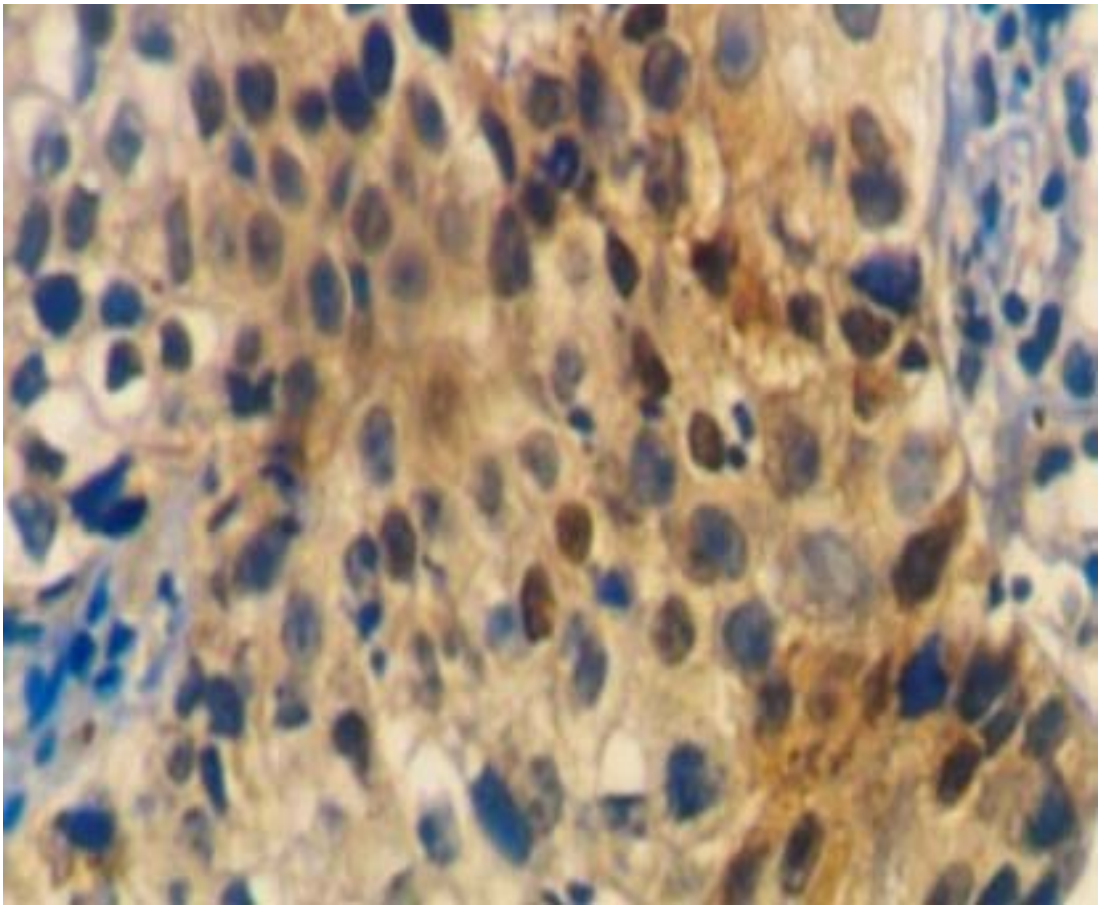
CYCLIN D1 EXPRESSION



SLIDE SHOWING NEGATIVE CONTROL



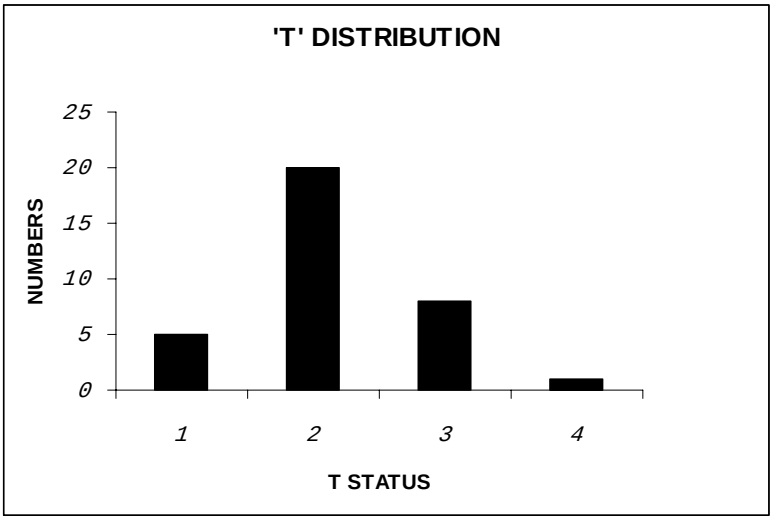
SLIDE SHOWING < 25 % EXPRESSION



RESULTS

Of the 37 cases analyzed and studied the T status distribution were :-

T status	Numbers	Percentage
1	6	16.2%
2	21	56.8%
3	9	24.3%
4	1	2.7%
Total	37	100%



The N status were

N	Numbers	Percentage
0	**30**	**81.1%**
1	**1**	**2.7%**
2	**6**	**16.2%**

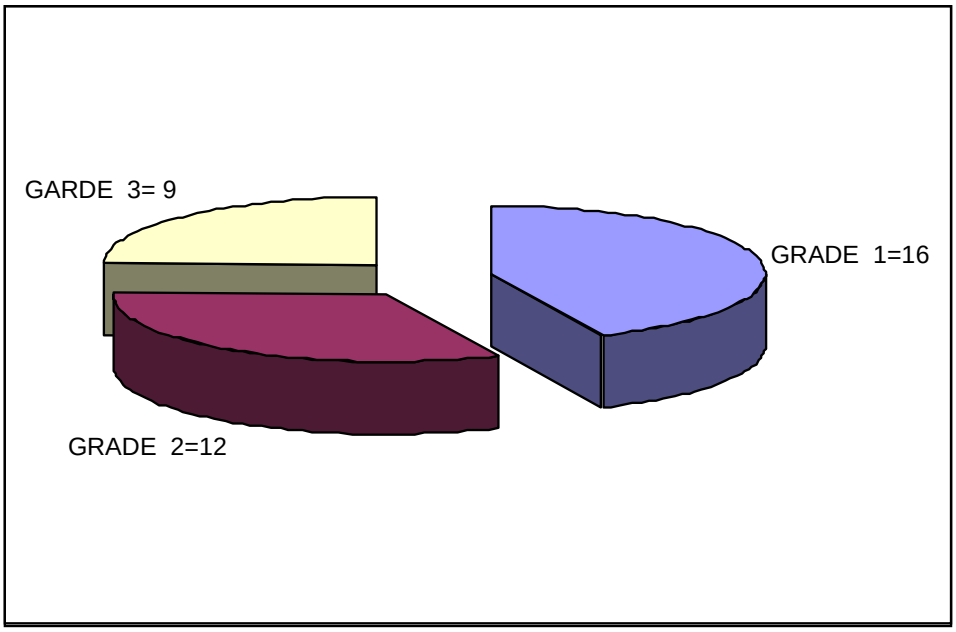
The grade distribution were

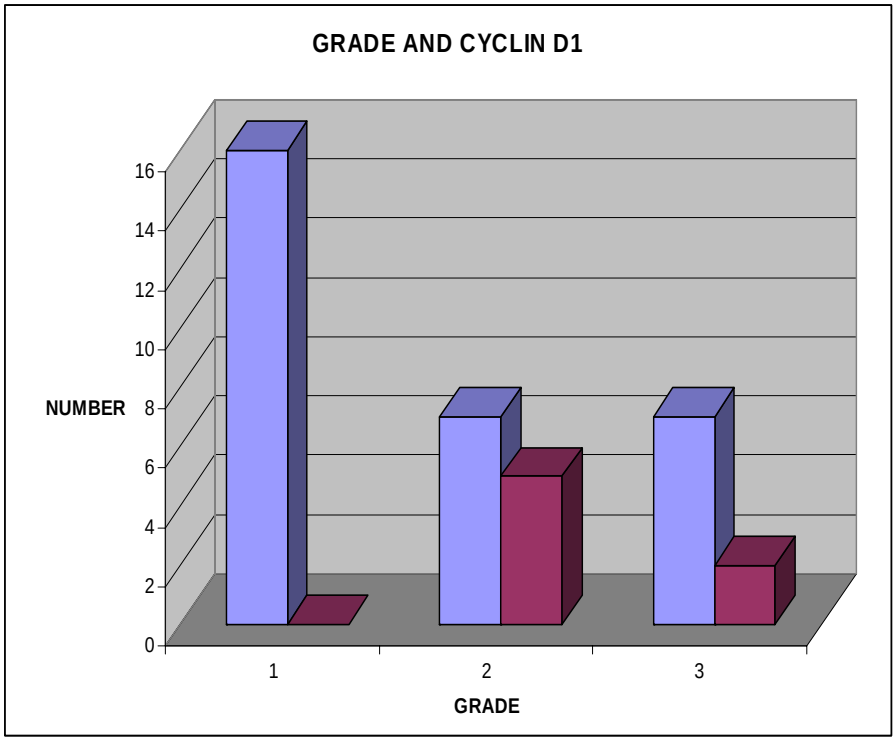
Grade	Numbers	Percentage
1	**16**	**43.2%**
2	**12**	**32.4%**
3	**9**	**24.3%**

Of these 37 cases that were followed up , there were 2 local recurrences (5.4%) that were salvaged and 10 regional recurrences(27%) . The cyclin D1 expression pattern in all cases were analyzed . Cyclin D1 expression pattern with respect to the T status showed 14.3 % of T 2, 44.4 % of T3 tumors expressed Cyclin D1 .None of the T1 or T4 tumors expressed .applying Chi square test did not show any statistical significance (*p* value 0.1244).

Cyclin D1 expression pattern with respect to grade of the tumor showed 41.7 % of grade II tumors and 22.2 % of grade III tumors overexpressed Cyclin D1 which was found to statistically significant (*p* value 0.0197) Even though the recurrence pattern and the Cyclin D1 correlation showed Cyclin D1 over expression only in 18.9% which statistically not significant (*p* =0.270) there was trend towards increased recurrence in those tumors that were over expressing Cyclin D1.

GRADE DISTRIBUTION





DISCUSSION

Cyclin D1 is one of the molecular markers that is over expressed in the actively dividing malignant epithelial cells. Most of the epithelial cancers as described before, over express Cyclin D1 .However there are only very few studies done to know the significance of this over expression in penile cancers. In our study we attempted to find the significance of this over expression with respect other clinico pathological factors.

In our study the cases chosen are highly selected with the criteria which was described above. Even though this may result in selection bias , the complete details are available only for those chosen cases .

Search of literature didn't show any reports regarding the cut off values for the scoring system of the expression. However there reports using > 5% of the stained cells as positive in cervical cancers. Using the same principle, our slides were scored .The normal squamous cells did not show positivity in none of our cases.

Analyzing Cyclin D1 expression with the T status there was no statistically significant difference between the expression patterns .The increasing T status did not show corresponding over expression of Cyclin D1 .This may be explained by the fact that the most of our patients present late .

With regard to the nodal status again the over expression did not show any significant correlation. However 16 % of the cases with N2

did show over expression .Since the number of cases analysed is small , a large study may throw light on the correlation between Nodal disease and Cyclin D1. All the patients who have their nodes over expressing Cyclin D1 also had their primary over expressing Cyclin D1.

With respect to the grade, of the 37 cases analysed there were 16 cases of grade I tumors, 12 cases of grade II tumors and 9 cases of grade III tumors. None of the cases of grade I tumor over expressed cyclin D1 .Five cases (41 %) of grade II tumors over expressed Cyclin D1. However only 22% of grade III tumors over expressed Cyclin D1 . Even though this showed statistically significant p value, the grade 3 tumors only 22 % over expressed which is paradoxical when compared to the grade 2 tumors. Since the number of cases studied was less and the grade of these tumors were analysed by different pathologists more standardization and inclusion of large numbers of cases may produce much more statistically significant values.

Even though the Cyclin D1 expression in recurrent tumors did not reach statistically significant value (p= 0.270), there is definite trend towards recurrence in tumors that over express Cyclin D1.a sudy with large number of patients in the future may show a significant correlation .

In some tumors there is a increased cyclin D1 RNA and/or protein without apparent gene amplification, suggesting that other cellular genes (such as the retinoblastoma gene) may impact on the protein expression of cyclin D1,[57] although all the mechanisms have not yet been satisfactorily elucidated. .In breast cancers it seems that cyclin D1 is more important in node positive,[74] well differentiated, and

particularly lobular, varieties than other types of invasive breast cancer.[91-93]The role of cyclin D1 in ductal carcinoma in situ (DCIS) [98], high grade lesions [99] were more likely to show gene amplification but demonstrated lower percentages of nuclei expressing cyclin D1 protein than low grade lesions, which suggests that mechanisms other than gene amplification may be responsible for increased cyclin D1 protein. In this situation, assessment of cyclin D1 protein in combination with pRB may provide more useful information.[100-102] The over expression of cyclin D mRNA, determined by in situ hybridization, was able to distinguish DCIS from atypical ductal hyperplasia and other lesions associated with a low risk of progression to invasive carcinoma[103].

35% to 64% of head and neck squamous carcinomas [106-111] (squamous carcinomas in the oral cavity, nasopharynx, pharynx, hypopharynx, and larynx) show over expression of cyclin D1 and/or CCND1 amplification. Over expression of cyclin D1 in the initial surgical specimens corresponds not only with more frequent recurrence [109] but also with more advanced disease, lymph node involvement, and reduced overall survival. [107] [110], over expression of cyclin D1 protein in resected material from head and neck squamous carcinomas was found to be an independent prognostic factor [111] In esophageal cancers, results suggest that cyclin D1 may play an important role in carcinogenesis and that cyclin D1 over expression may be a useful prognostic factor [120] Abnormalities of cyclin D1 are also common in both vulval and cervical squamous cell carcinomas. [135] At these sites, cyclin D1 appears to inactivate pRB in a similar manner to oncogenic human Papilloma virus genotypes. Thus, it seems that in vulval and cervical squamous

carcinomas, human papilloma virus proteins can circumvent cellular requirements for cyclin D1 [136] or vice versa.

HPV which is the commonest etiological agent for carcinoma penis, especially in developing countries may result in gene expression abnormalities of cyclin d1 geneswhich may account for the over expression .More studies with correlation to hpv genome studies Cyclin D1 gene and other genes like Rb gene , p53 gene and their expression patterns are required to arrive at a definite conclusion .

CONCLUSIONS

CYCLIN D1 IS OVER EXPRESSED IN PENILE CANCERS

HIGH GRADE SQUAMOUS CELL CARCINOMAS OVER EXPRESS CYCLIN D1, WHICH IS STATISTICALLY SIGNIFICANT

EVEN THOUGH CYCLIN D1 EXPRESSION IN RECURRENT TUMORS IS NOT STATISTICALLY SIGNIFICANT, THERE IS A TREND TOWARDS OVER EXPRESSION IN RECURRENT TUMORS

CYCLIN D1 EXPRESSION DOES NOT SHOW ANY CORRELATION WITH ‘T’ STATUS AND ‘N’ STATUS.

FUTURE DIRECTIONS

1. CYCLIN D1 MAY BE USED AS A PREDICTIVE AND PROGNOSTIC MARKER OF METASTASES , HOWEVER LARGE STUDY IS REQUIRED TO SHOW ITS DEFINITE SIGNIFICANCE
2. MORE WORK IS NEEDED TO DETERMINE THE EXACT ASSOCIATION BETWEEN CYCLIN D1 AND HPV VIRUS.
3. EXPRESSION OF CYCLIN D1 IN HIGH RISK NODE NEGATIVE PATIENTS MAY BE AN INDICATION FOR ELCTIVE NODAL DISSECTION.
4. MORE WORK NEEDS TO BE DONE ON THE ROLE OF CYCLIN D1 IN PRECANCEROUS PENILE LESIONS.
5. EXPRESSION PATTERNS IN HIGH GRADE LESION MAY GUIDE THE ROLE OF ADJUVANT THERAPY.
6. GENE STUDIES ON THE EXPRESSION OF CCND1 GENES.
7. CORRELATION BETWEEN CYCLIN D1 AND HPV NEEDS TO BE IDENTIFIED.

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